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A Comprehensive Approach to Assess Feathermeal as an Alternative Protein Source in Aquafeed

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ABSTRACT

The effect of partially replacing fishmeal in aquafeed with feathermeal (FTH) at three levels (0%: FTH0, 8%: FTH8, 24%: FTH24) and two extrusion temperatures (100 and 130 °C) were evaluated in rainbow trout (*Oncorhynchus mykiss*) with respect to growth performance, metabolism response, and oxidative status of the feed proteins. Multivariate data analyses revealed that FTH24 correlated positively with high levels of: oxidation products, amino acids (AA) racemization, glucogenic AAs level in liver, feed intake (FI), specific growth rate (SGR), and feed conversion ratio (FCR); and low AAs digestibility. Both FI and SGR were significantly increased when 8 and 24% feathermeal was included in the feed extruded at 100 °C, while there was a negative effect on FCR in fish fed FTH24. In conclusion, higher oxidation levels in FTH24 may give rise to metabolic alterations while lower levels of FTH may be considered as fishmeal substitute in aquafeed for rainbow trout.

Keywords: fishfeed; fishmeal; feathermeal; extrusion; oxidation; metabolite; growth performance; rainbow trout; *Oncorhynchus mykiss*

INTRODUCTION

In recent years, the soaring global demand for protein has boosted commercial fish farming dramatically. Hence, global production of aquafeeds is increasing and expected to reach 71 million metric tonnes by 2020, corresponding to an increase at an average rate of 11 percent per year [1-2]. Feed costs are a significant part of the total production costs, mostly due to high cost of fishmeal [2-3]. However, there are several ways to reduce the fishmeal proportion in aquafeed. Increasing attention has been paid to utilization of more economically and environmentally sustainable alternative protein sources to reduce production costs [4-6]. Feathermeal (FTH) is becoming attractive due to high supply options, low costs, its high content of protein and essential amino acids (AAs), and the lack of anti-nutritional factors [7]. Recently, the potential of utilizing FTH in extruded fish feed was investigated and showed promising results [8]. Overall, it was found that the formation of oxidation products and heat-induced cross-links increased with a high inclusion level of feathermeal (24%). However, it was also found that an inclusion of 8% FTH in the feed resulted in the highest *in vitro* digestibility [8]. These preliminary findings underlined that the relationship between chemical and physicochemical changes of proteins and digestibility is more or less straightforward; hence, the biochemical and biological effects, especially the bioavailability, must be taken into account upon evaluating protein replacement. In previous studies on replacement of fishmeal with feathermeal [9-11] there has, to the best of our knowledge, been no focus on the relationship between feed protein chemical changes and fish biological performance.

During extrusion, feed ingredients undergo extensive heat treatments at high-pressure conditions. Therefore, heat sensitive AAs such as methionine, lysine, and tryptophan may suffer from oxidative damages, which may reduce the digestion or absorption of nutrients and consequently affect growth performance and even induce toxicity [12-13]. Knowledge

about the effect of extruded proteins on the chemical and biological characteristics in relation to aquafeed is, however, scarce. The current study was therefore conducted to provide more knowledge about the interactions between extrusion and fishmeal replacement with FTH and the chemical effects in feed and biological effects in rainbow trout (*Oncorhynchus mykiss*). Based on recent results (own unpublished data), feed with inclusion levels of 8% (the best candidate) and 24% (worst-case scenario) were produced in industrial settings at two different extrusion temperatures (100 and 130 °C). Extrusion processing effect on proteins was monitored as protein oxidation products, amino acid digestibility, and amino acid racemization (AAR). Furthermore, the effects of protein changes on fish growth performance and liver and plasma metabolites were monitored.

MATERIALS AND METHODS

Extrudate and Feed Production

Feed was produced and extruded by Biomar A/S (Biomar A/S, Tech Center, Brande, Denmark). A feed production experiment was designed according to a 3 × 2 factorial model with three feathermeal inclusion levels (0, 8, and 24%) and two extrusion temperatures (100 and 130 °C). The feeds were formulated to have similar level of macronutrients, to be iso-nitrogeneous, iso-energetic by balancing with wheat flour, and to meet rainbow trout requirements [14]. The content of lysine, histidine, methionine, and tryptophan was maintained constant by adding L-lysine HCl, L-histidine, DL-methionine, and L-tryptophan respectively, while phosphorus was optimized by adding mono-calcium phosphate. Yttrium oxide was added as internal marker. Recipes and the chemical composition of fishmeal and feathermeal, the meal mixes, the extrudates, and experimental feed after oil coating are given in Table 1.

Feed ingredients were milled with a hammer mill to pass through a 0.75 mm screen. The formulation mixtures were subsequently extruded in a five-section twin-screw extruder (Clextral BC 45 extruder, Clextral, France) equipped with a 2.4 mm die. Moisture content of the dough during extrusion was set at 25%. Following extrusion processing, the extrudates were dried and coated by fish- and rapeseed oil using a vacuum oil pump. Samples labelled 'extrudate' were sampled immediately after the extrusion process and stored in closed plastic containers at 4 °C until analysis for oxidation and heat-induced products. Samples labelled 'feed' (6 codes: FTH0/T100, FTH0/T130, FTH8/T100, FTH8/T130, FTH24/T100, and FTH24/T130) refer to the extrudates after drying and oil coating and were stored in bags at 4 °C until used in the fish trials.

Protein Extraction and Determination of Solubilized Proteins

Samples (50 mg) of meal mixes and extrudates were shaken for 4 h in 10 mL of 6 M guanidine hydrochloride (GuHCl). Samples were then centrifuged for 1 min at 1000 rpm, and the supernatants (protein solutions) were collected for analysis of solubilized protein content and oxidation products. The solubilized protein content of the raw meal mixes and extrudates was determined by a bicinchoninic acid (BCA) assay kit (Pierce, Bonn, Germany) according to the manufacturer instruction using the microplate procedure (25 µL sample/200 µL BCA reagent; 37 °C/30 min). Bovine Serum Albumin (BSA from Sigma, Munich, Germany) was used as protein standard. The absorbance of the solution was measured using a spectrophotometer at a wavelength of 562 nm. Each sample was assayed in triplicate.

Oxidation and Heat-induced Changes

All analyses of heat-induced changes of proteins were performed on the extrudates. The following measurements of oxidation products were all based on soluble proteins (ca. 0.6 mg/mL) obtained from protein extraction in 6 M GuHCl as mentioned above and selected wavelengths from following references;

Protein hydroperoxides (PHP): the content of PHP was obtained by mixing an aliquot of the protein solution with a xylenol orange and ammonium ferrous reagent following incubation and measurement of absorbance at 560 nm [15] using a spectrofluorometer (Synergy 2 spectrofluorometer (BioTek, Winooski, VT, USA)). The standard curve for quantification (μmol PHP/mg protein) was based on hydrogen peroxide (0 to 30 μM).

Total carbonyl: the content of total carbonyl (arbitrary intensity units (AU)/mg protein) of an aliquot of the protein solution was obtained by fluorescence detection with excitation at 350 nm [16] and emission intensity at 447 nm in non-transparent microtiter plates (96-Corning-Costar (Lowell, MA, USA)) on a Perkin Elmer LS 50B spectrofluorometer (PerkinElmer, Massachusetts, United States).

N-formylkynurenine (NFK): the content of NFK (arbitrary intensity units (AU)/mg protein) of an aliquot of the protein solution was obtained by fluorescence detection with excitation at 330 nm [15, 17] and emission intensity at 449 nm in non-transparent microtiter plates (96-Corning-Costar (Lowell, MA, USA)) using a Perkin Elmer LS 50B spectrofluorometer (PerkinElmer, Massachusetts, United States).

Schiff base products: the content of Schiff base products (arbitrary intensity units (AU)/mg protein) of an aliquot of the protein solution was obtained by fluorescence detection with excitation at 345 nm and emission intensity at 449 nm [18-19] in non-transparent microtiter plates (96-Corning-Costar (Lowell, MA, USA)) using a Perkin Elmer LS 50B spectrofluorometer (PerkinElmer, Massachusetts, United States).

Lanthionine and furosine: Quantification of lanthionine and furosine was carried out following acidic hydrolysis of the extrudates. Briefly, an amount of sample corresponding to 10 mg protein was suspended in 1 mL 6M hydrochloric acid (HCl) and incubated for 24 hours at 100 °C. HCl was subsequently removed by purging with nitrogen gas, and the dried sample was resuspended in 1 mL MilliQ water and sonicated for 5 min in water bath (Marshall Scientific Branson 1210 Ultrasonic Cleaner) at room temperature. The samples were centrifuged (10 min, 19000 g, 4 °C) and supernatants were diluted 1:50 in MilliQ water containing 1 µg/mL internal standard deuterated lysine (L-Lysine-4,4,5,5-d4 hydrochloride, Sigma Aldrich). Diluted samples were centrifuged (10 min, 19000 g, 4 °C) and 10 µL of the supernatants were injected into a LC-MS/MS system. Quantification was performed via an RP-UPLC (Thermo-Scientific) featuring a C18 column (Phenomenex Aeris XB-C18. 1.7 µm particle size, 150 x 2.1 mm) coupled with mass spectrometer (Q-Exactive Orbitrap) using electrospray ionization in positive mode. The analytes were eluted from the LC column using a 27-min method with aqueous (A) and organic buffers (100% acetonitrile, B) both containing 5 mM perfluoropentanoic acid. The method was designed as follows: 100% A (0 to 5 minutes), 100 to 50% A (5 to 15 min), 50 to 100% B (15 to 17 min), 100% B (17 to 22 min), 100% B to 100% A (22 to 24 min) and 100% A (24 to 27 min) at a constant flow rate and oven temperature of 0.25 mL/min and 40 °C, respectively. Direct injection of standard solutions in the mass spectrometer was used to determine ionization source parameters (auto-tuning).

Peaks were identified and quantified by monitoring the specific m/z ratios for each analytes (Table 2). Processing and quantification was performed using the ThermoScientific Xcalibur software. A standard curve (5 to 10000 ng/mL) was derived for every standard prior to sample analysis, using for every point the analyte/internal standard peak area ratio (PAR). The internal standard was present in each point of the

curve and for each analyte at the same concentration (1 µg/mL). The calculated furosine and lanthionine concentrations in the samples were then compared to the known protein concentration of each material and expressed in µg/mg protein.

Amino Acid Racemization

The method for measuring amino acid racemization (AAR) was based on Tojo et al. (2012) [20], which combines derivatization using Marfey´s reagent (2,4-Dinitro-5-fluorophenyl; FDAA, Sigma Aldrich 71478) with separation and quantification of D- and L-amino acids by HPLC. D-AAs derivatized with Marfey's reagent exhibit strong intramolecular bonding, which reduces their polarity relative to the corresponding L-amino acid derivates. Consequently, the D-derivates are selectively retained on reverse phase columns and elute later than the corresponding L-derivates.

Approximately 10 mg of grinded feed were added to hydrolysis tubes (Thermo Scientific 29571) in addition to 1.7 mL 6 *N* HCl containing 0.2% phenol (w/v). Air/oxygen was removed by flushing the tubes with nitrogen gas followed by application of vacuum (alternating 3 times, 30 sec each) and tightening the lid under vacuum. The samples were hydrolysed for 24 hours at 110 °C. Nor-Leucine (Sigma Aldrich N8513) was added as an internal standard for estimation of recovery. The hydrolysed samples were transferred to glass tubes and the HCl evaporated in a vacuum-concentrator (CentriVap, VWR 531-0224). The samples were re-suspended in 33% acetonitrile and filtered by 0.2 µm centrifugation filters (VWR 516-0234) and could hereafter be derivatized with 1% FDAA in acetone, according to Thermo Scientific online protocol (MAN0016377), using 50 µL for standards or feed samples. As derivatized samples are rather unstable, they were analyzed immediately after derivatization. Identification and quantification was performed

by means of an uHPLC system (Flexar FX-10, PerkinElmer Inc., Waltham, MA, USA) using gradients of 50 mM trimethylamine-phosphate buffer, pH 3.5, containing either 10 or 40% acetonitrile (mobile phases A and B, respectively). Standard curves were prepared using a standard mix of L- amino acids (Sigma Aldrich A9781) added the D-isoforms (Sigma Aldrich) of methionine (M9375), lysine (L8021), threonine (T8250), phenylalanine (P1751) and valine (855987). The chromatographs for the different samples were analyzed using the CHROMORA FLEXAR v3.2.0 4847 software (PerkinElmer Inc.). The amount of D-AA per kg feed were subsequently calculated, taking recovery of nor-leucine into account. The degree of AAR of each individual AA was calculated as:

Degree of AAR= D/(D+L); where D and L refer to the two isoforms of the amino acid.

Fish trial

An 8-weeks fish performance trial was carried out in a recirculating freshwater aquaculture system at the Biomar Research Center in Hirtshals, Denmark. The trial was carried out in accordance with EU legislation and Danish Animal Welfare Regulations. All six feed codes (FTH0/T100, FTH0/T130, FTH8/T100, FTH8/T130, FTH24/T100, and FTH24/T130) were fed to triplicate tanks containing 90 rainbow trout each with a start weight of 111.2 ± 2.60 g. Fish were fed ad libitum every 6h each day, and uneaten pellets were collected and weighted. Water temperature (12 °C), oxygen (>92%), and a light:dark ratio (16:8 h) were kept constant for the duration of the trial. Upon finalizing the feeding period, the fish were anaesthetized, gently cleaned with soft tissue and weighed, stripped for feces, and plasma and liver samples were obtained. Growth performance parameters including the specific growth rate in % day^{-1} (SGR; 100*(ln final weight – ln initial weight)/feeding days), feed conversion ratio (FCR, dry feed intake/wet weight gain), and

daily feed intake in % day^{-1} (FI; 100*((daily feed load - daily feed loss)/feeding days) were calculated for each replicate at the end of the study.

In vivo Amino Acid Digestibility

The amino acid composition of the feed and stripped feces from the rainbow trout was analyzed according to ISO 13903 [21], and the apparent digestibility (ADC) of the amino acids [22] was calculated as follows: $ADC_i = (1-(F_i/D_i \times D_y/F_y)) \times 100$; where F_i and D_i refer to the percentages of the amino acid (i) in the feces and diet, respectively, and F_y and D_y refer to the percentage of yttrium (y) in the feces and diet, respectively.

^{1}H NMR-based Metabolomics Analyses

Sample Preparation for Metabolomics Study of Plasma and Liver

Preparing fish plasma samples for ^{1}H NMR analyses were carried out as described previously [23], with slight modification. Briefly, Nanosep centrifugal filters with 3 kDa cut-off (Pall Life Science, Port Washington, NY, USA) were washed three times with MilliQ water (2000 g, 12 min, 30 ºC) to remove glycerol from the filter membrane. For plasma analyses, pooled samples from 5 fish per tank were used. This included mixing, 100 µL of each plasma sample into one tube (total= 500 µL) and centrifuging at 13000 g at 4 ºC. 200 µL of the pooled plasma samples were subsequently mixed with 350 µL of deuterium oxide (D_2O) and 50 µL D_2O containing 0.05 wt% of sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as internal standard. The pooled plasma samples were prepared in duplicates.

Fish liver samples were extracted and prepared for ^{1}H NMR analyses following the method described previously [24], with few modifications. Hence, 20 mg of lyophilized, grinded homorganic liver powder was whirl-mixed in 3 steps of 1 min duration each: first

in 300 µL ice-cold methanol, then in 300 µL ice-cold chloroform and third in 300 µL ice-cold water. The samples were placed on ice for 10 min between each step and finally stored at 4 °C overnight for separation. The following day the samples were centrifuged (30 min, 1400 g, 4 ºC) (Eppendorf centrifuge 5417, USA), and following phase separation the aqueous and chloroform supernatant was collected in separate tubes. The collected aqueous phase samples were dried using an evacuated centrifuge (Eppendorf Concentrator Plus, Germany) for approximately 3 hours and re-dissolved with 550 µL D_2O, 25µL MilliQ water and 25 µL D_2O containing 0.05 wt% TSP. The chloroform phase samples were dried for approximately 1 hour and re-dissolved in 575 µL $CDCl_3$ (99.96 atom% D) and 25 µL $CDCl_3$ containing 0.05 wt% TSP. The liver samples were prepared in quintuplicate (5 individual fish per tank and diet). The plasma and liver samples were subsequently analyzed with ^{1}H NMR in 5 mm NMR tubes (Bruker Spectrospin Ltd, BioSpin, Karlsruhe, Germany).

^{1}H NMR Spectroscopy, data processing and identification of the signals

The plasma and liver samples were analyzed with a Bruker 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) using zgpr pulse sequence at 25 ºC with 64 scans, a spectral width of 7,288 Hz collected into 32,768 data points, an acquisition time of 2.24 sec, and an interscan relaxation delay of 5 sec. The ^{1}H NMR spectra for the chloroform liver phase was obtained using zg30 pulse sequence (Bruker) at 20 ºC with 64 scans and 65,536 data points over a spectral width of 12,335 Hz. Acquisition time was 2.65 sec and relaxation delay 1sec.

All data were processed using the Bruker Topspin 3.0 software (Bruker) and Fourier-transformed after multiplication by line broadening of 0.3 Hz. The spectra were

237 referenced to standard peak TSP (chemical shift 0 ppm), phased and baseline corrected.
238 Each NMR spectrum was integrated using Matlab R2011b (Mathworks, USA) into 0.01
239 ppm integral region (buckets) between 0.5-9.5 ppm and 0.8-9.0 ppm for aqueous liver
240 phase and plasma extracts, respectively, in which area between 4.7 and 5.0 ppm (4.7 and
241 5.15 ppm for plasma) corresponding to water signal was excluded and for chloroform
242 liver samples between 0.6 and 5.5ppm. For the aqueous and chloroform liver phase each
243 spectral region was normalized to the intensity of internal standard (TSP) for quantitative
244 measurements and for plasma samples was normalized to the sum of total area. The
245 chloroform samples were not analyzed further. For the plasma and aqueous liver samples
246 the ChenomX NMR Suite version 8.1 profiler (ChenomX Inc, Edmonton, AB, Canada)
247 was used to identify and quantify compounds. A total of 55 metabolites in the plasma and
248 aqueous liver phase were identified by overlapping with standard spectra, and their
249 concentrations were expressed in μmol/mg for liver and μmol/L for plasma. Assignments
250 of the ^{1}H NMR signals were carried out using ChenomX NMR Suite 8.1 library
251 (ChenomX Inc), the Human Metabolome Database (www.hmdb.ca) and previous
252 literature [24-26], and confirmed with 2D-NMR in case of multiplicity.

253 **Data Analyses**

254 The Simca-P software (version 14.0; Umetrics, Umeå, Sweden) was applied for
255 multivariate data analyses of the absolute concentrations of the metabolites. All variables
256 were “unit variance” (UV)-scaled. Principal component analyses (PCA) was used to get a
257 first overview of the data and search for outliers. Outliers were observed using PCA-
258 Hotelling T^2 Ellipse (95% confidential interval (CI)). Data on protein oxidation
259 compounds, fish growth performance parameters, and metabolites were subjected to one-
260 way and two-way analysis of variance (ANOVA) and Duncan's multiple range tests to
261 compare the effects of different experimental conditions examined and their main effects

and interactions. An independent Student's t-Test analysis was performed to find out whether significantly different liver and plasma metabolites existed between fish fed the low (FTH0) and high (FTH24) level of feathermeal feeds. Statistical analyses were carried out using the IBM SPSS STATISTICS statistical program, (version 22.0, IBM Corporation, New York, USA). Differences were considered significant when $P < 0.05$ unless otherwise indicated.

RESULTS

In the present study, we investigated the effects of two extrusion temperatures (100 and 130 °C) and three feathermeal inclusion levels (0, 8, and 24%) on the chemical properties of proteins in the extrudates (i.e., protein oxidation products and AAR) and on fish growth performance (FCR, FI, and SGR) and *in vivo* AAs digestibility. In order to explain the underlying mechanism of how the feed parameters affected the growth performance, liver and plasma metabolites in individual rainbow trout were also examined following a 8 weeks feeding study. Hence, all results were subjected to a multivariate data analysis to assess the overall relationships. In addition, the most common oxidation products and growth performance results are presented and discussed in details, while other results are presented in the supplementary material.

Growth performance

In general, all feeds were well accepted by the fish and the average body weight increased from 111.2 ± 2.6 g to 212.2 ± 10.9 g during the 8 weeks of feeding. There was a significant main effect of the level of FTH on the growth parameters (FI, SGR, and FCR),

while no main effect of the extrusion temperature was found (Table 3). Meanwhile, there was a significant interaction between the extrusion temperature and FTH level on FI and SGR (Table 3). A significantly higher FI was seen in fish fed feathermeal diets (FTH8 and FTH24) extruded at 100 °C compared to the control group (FTH0), while no similar effect was observed for diets processed at 130 °C (Fig. 1A). The feed intake in the control group (FTH0) was significantly higher when fed the diet extruded at 130 °C compared to 100 °C (Fig. 1A). The SGR of fish fed the FTH0 diet extruded at 100 °C was significantly lower than that of fish fed any of the other experimental feed (Fig. 1B). At the same time, replacing fishmeal with a high level of feathermeal (24%) significantly increased the FCR compared to the other groups independently of the extrusion temperature (Fig. 1C).

Protein Oxidation and Amino Acid Racemization

The PCA analysis (Fig. 2B) showed that the protein oxidation products, e.g. total carbonyl, NFK, and Schiff base grouped together, indicating a similar variance of the data. Hence, the oxidation and heat-induced products including PHP, carbonylation, and lanthionine together with methionine racemization (Fig. 3) serve as representative markers of changes in the primary protein structure following extrusion processing. For a detailed overview of the other protein degradation products (NFK, Schiff base, furosine, and specific AA racemization) the reader is referred to supplementary material (Fig. S1). The primary oxidation product, PHP, did not change significantly due to increased extrusion temperature to 130 °C irrespectively of the FTH inclusion levels (Fig. 3A). Only FTH24 showed a significant increase in PHP as an effect of increasing the extrusion temperature from 100 to 130 °C. Carbonylation is a measure of the protein oxidation propagation and,

as seen in Fig. 3B, increasing both the extrusion temperature and the feathermeal inclusion level resulted in a significant increase in the level of total carbonyls.

Regarding changes in the physical characteristics of the proteins, the amount of FTH had a significant effect on the formation of cross-links. The content of lanthionine was significantly higher in the extrudates with the highest level of feathermeal (FTH24) compared to that without feathermeal (FTH0), whereas no effect of processing temperature was found (Fig. 3C). Amino acid AAR can have a great impact on protein bioavailability and the degree of methionine racemization represents the physical changes due to AAR. Hence, increasing the level of feathermeal increased the degree of methionine racemization whereas no effect of processing temperature was found (Fig. 3D). The same pattern was observed for racemization of phenylalanine (Fig S1F) while not similar effect was observed for the other tested amino acids (i.e., lysine, threonine, and valine; Fig S1B, D, and G, respectively)

Correlation of Feed Variables with Growth Responses Variables

In order to compare the results presented in Fig. 1 and 3 (and S1), PCA modelling was carried out. The resulting PCA plot of the feed variables and growth performance data show that the model was principally able to separate the different feeds by the first two components with the first principal component (PC1) explaining 72% of the variance in the data matrix and the second PC (PC2) explaining 14% (Fig. 2A). The extrudates, feed characteristics and fish growth performance data clearly grouped by the feathermeal level (Fig. 2), while samples were not separated according to temperature (Fig. S2). In general, an increase in feathermeal correlated with an increase in FCR, SGR, and FI (Fig. 2B). Moreover, the highest level of feathermeal correlated positively with an accumulation of oxidation and heat-induced products in the extrudates (e.g. PHP, carbonyls, NFK, Schiff

base, lanthionine, and furosine) compared to extrudates with no or low inclusion of feathermeal (Fig. 2A, B). The lack of a similar dependency of FTH level and extrusion temperature on the specific AAR (Fig. 3D, Fig. S1 B, D, F, G) is seen from the scattering of these data in the PCA plot, though a high accumulation level of AAR was correlated with samples with feathermeal included, especially with FTH24 (Fig. 2B). Furthermore, high *in vivo* digestibility of amino acids correlated largely with a lack of feathermeal in the feed (Fig. 2B).

Correlation of Fish Metabolites (Liver and Plasma) with Growth Response Variables

A PCA was also used to examine the covariance between fish growth performance, metabolites (liver and plasma), extrusion temperatures, and feathermeal inclusion levels (Fig. 4 and S3). The PCA scores plot of the liver and plasma metabolites and growth performance data displayed group separation according to the feathermeal inclusion levels along PC1, explaining 21.6 % of variation, whereas PC2 explained 12.6% of variation (Fig. 4A). Hence, separation was not as confined as the protein changes (Fig. 2), but similar to the protein changes no separation was observed with respect to extrusion temperature (Fig. S3).

The variables important for the observed grouping included mainly AAs and organic acids (Fig. 4B, Table 4 & 5). Among the plasma AAs, phenylalanine, proline, valine, serine, tyrosine, leucine, and methionine correlated positively with the inclusion level of feathermeal. In contrast, plasma lysine and arginine were negatively correlated to the level of feathermeal (Fig. 4B). Only tyrosine, valine and phenylalanine were significantly different between dietary treatments following univariate statistics (Table 4). For the liver AAs, phenylalanine, arginine, methionine, valine, isoleucine, tyrosine, alanine, and

leucine were positively associated with fish fed FTH24, while liver lysine correlated positively with the control diet (FTH0). Detailed comparisons of liver metabolites are presented in Table 5. Furthermore, pyruvate level in the liver, plasma levels of lactate and glucose, FCR, SGR, and FI were positively correlated with FTH24 (Table 4 and Fig. 4B). Creatinine, creatine, acetate, NAD+, ATP, ADP, in liver were positively correlated with the control group (FTH0).

Significantly Different Plasma and Liver Metabolites between FTH0 and FTH24

The largest effects on plasma and liver metabolites (Fig. 4A) were seen between fish fed the high feathermeal diet and fish fed the control diet. A high inclusion of feathermeal resulted in a decrease in plasma creatinine, dimethylamine, trimethylamine, trimethylamine-n-oxide, n-methylhydantoin, and an increase in plasma phenylalanine, valine, methionine, tyrosine independently of the extrusion temperature (Table 4). In contrast, liver metabolites were with a few exceptions more affected by the extrusion temperature. Hence, a high inclusion of feathermeal in the diet extruded at a high temperature resulted in a decrease of creatine, creatinine, NADP+, taurine and threonine, whereas the low extrusion temperature of the same diet resulted in an increase in isoleucine, phenylalanine, valine and beta-alanine (Table 5).

DISCUSSION

The global shortage of fishmeal as a primary source of protein forces the aquafeed industry to use unconventional protein ingredients in formulated aquafeed [27]. A large number of poultry-industry waste materials such as feathermeal can potentially be used. However, high concentrations of sulfur-containing AAs that are more susceptible to oxidation than fishmeal makes feathermeal questionable with respect to digestibility.

379 Hence, reduced fish growth [28-30] and altered immune response [31] have been reported when
380 oxidized feeds have been used in aquaculture.

381 In the present study, the extent of protein oxidation in the extruded feed was found to be a
382 function of both extrusion cooking temperature and feathermeal inclusion level. Hence,
383 mildly treated samples (100 °C) had fewer protein oxidation products compared to the
384 more harshly treated samples (130 °C). Furthermore, the degree of racemization of the
385 examined AAs correlated positively with the FTH inclusion, being highest in FTH24. The
386 higher levels of AAR and protein oxidation products in feed with feathermeal proteins
387 might be attributed to the transformation of free radicals formed from sulfur-containing
388 AAs oxidized during extrusion cooking and turned into other AAs [32].

389 The accumulation of lanthionine in feed with high FTH inclusion did not seem to be
390 affected by the extrusion temperature. In comparison, previous studies have shown that
391 heat treatment has a significant effect on the formation of unnatural AAs, particularly
392 lanthionine, leading to a reduction in protein digestibility [33-34]. The results in the current
393 study are consistent with the observation that oxidative cross-linking occurring in
394 feathermeal can reduce AAs digestibility *in vivo*. The digestibility of proteins typically
395 decreases when the ratio of AAR increases due to the stereospecificity of proteinases and
396 peptidases [32]. Furthermore, the positive correlation between FCR and oxidation products
397 found (Fig. 2) indicates that protein, being one of the most valuable components of the
398 feed from a nutritional standpoint, can be made partly unobtainable to the fish due to heat-
399 induced damages. Hence, the levels of oxidation products and AAR in the feed combined
400 with reduction in *in vivo* AAs digestibility and higher FI in fish fed FTH24 all suggest that
401 the heat-induced damages on proteins plays a significant role in energy demanding
402 process.

In aquafeed, an optimal proportion of all essential AAs is required for efficient protein utilization and growth of the fish [35]. Feathermeal is deficient in several AAs including methionine, lysine, histidine, and tryptophan [36]. In the present study, these essential AAs were therefore supplemented as free AAs to diets containing FTH (Table 1). It has previously been reported that lysine, sulfur-containing amino acids, and the indole ring of tryptophan are the AAs most susceptible sensitive to oxidation [37-38]. Consistent with this, high level of protein oxidation was observed in the feed containing FTH supplemented with free essential AAs. Similar to the current study, a previous study has also shown that FCR increases when diets are supplemented with free AAs [39]. The higher FI in fish fed high amounts of FTH may thus be a reflection of an increased energy demand deriving from de novo protein synthesis from AAs damaged during extrusion cooking or catabolic expenses associated with deaminating and excreting the damaged AAs. Furthermore, increased energy demand due to consumption of oxidized proteins might also be the reason for higher FI in the control group fed the diet extruded at 130 °C, in which higher protein oxidation were found compared to the diet extruded at 100 °C.

A high inclusion level of feathermeal resulted in a higher hepatic pyruvate level. Pyruvate can be produced from glucose via glycolysis in the cytosol. It usually penetrates the mitochondria and is converted to acetyl Co-A which enters the tricarboxylic acid (TCA) cycle generating energy in form of Adenosine triphosphate (ATP) [40]. An increase of pyruvate in the liver may thus indicate that its use in the TCA cycle was somehow affected. Consistent with this, lower levels of ATP correlated with a high dietary inclusion level of feathermeal, indicating that the liver cells were energy limited. Furthermore, the high correlation between FTH24 and the levels of lactate in both liver and plasma samples corroborate that pyruvate did not efficiently enter the TCA cycle, explaining that there was no need to deplete lactate from blood. Consistent with these results, a higher glucose

428 level in the plasma correlated with a high inclusion level of feathermeal. A high glucose
429 content in the plasma of fish fed feed containing high levels of feathermeal may be related
430 to an impaired TCA cycle leading to a reduction in the glycolytic activity and
431 consequently an insufficient transfer of glucose from the blood stream into the body cells.
432 The low levels of NAD^+ in the liver of fish fed feed containing high levels of feathermeal
433 are consistent with this hypothesis. Under normal conditions, NAD^+ promotes the release
434 of energy from pyruvate via the TCA cycle [40]. Furthermore, a lack of pyruvate in the
435 mitochondria would activate glutamine metabolism to ensure a persistent TCA cycle
436 function [41]. Consistent with this, the low concentrations of glutamine in the liver in the
437 present study correlating with a high inclusion level of feathermeal might indicate
438 glutamine depletion due to a lack of pyruvate.

439 It is well known that dietary ingredients can be reflected in fish tissues or biofluids. In the
440 present study, the hepatic levels of leucine, isoleucine, tyrosine, valine, methionine,
441 arginine, and phenylalanine correlated positively with fish fed a high inclusion level of
442 feathermeal. All of the above mentioned AAs are involved in energy metabolism [24, 40].
443 The increase in the concentration of these AAs in the liver thus indicates that they were
444 inhibited from entering the TCA metabolic pathway and thereby hindered from generating
445 energy, potentially explaining the higher FI in fish fed FTH24 compared to the control
446 group. Furthermore, tyrosine synthesized from the essential AA phenylalanine is a
447 precursor for thyroid hormones and neurotransmitters [42]. Thyroid hormones play an
448 important role next to energy metabolism and protein synthesis, and indirectly affect the
449 feed intake, feed conversation efficiency and growth performance. Thus, higher tyrosine
450 (plasma) and phenylalanine (liver and plasma) levels in fish fed FTH24 may indicate a
451 thyroid promoting effect of the feathermeal diet due to higher energy demands,
452 subsequently leading to the observed increased feed intake.

453 Trimethylamine-n-oxide (TMAO) has protein-stabilizing capabilities and prevents
454 oxidative damages [43-44]. High TMAO levels can be obtained either from the diet or by
455 endogenous biosynthesis from the trimethylamine moiety of choline [45]. In the current
456 study, we observed a lower level of TMAO with higher inclusion of feathermeal
457 independently of the extrusion temperature in plasma and for high extrusion temperature
458 in the liver. The observation might be explained with the lower levels of TMAO found in
459 the diets (i.e., FTH0 vs. FTH24 for mild extrusion: 0.56 ± 0.06 μM/mg vs. 0.44 ± 0.02
460 μM/mg, respectively; for harsh extrusion: 0.60 ± 0.00 μM/mg vs. 0.50 ± 0.00 μM/mg,
461 respectively).

462 In summary, the results in the current study showed that protein oxidation increases as a
463 function of the extrusion temperature and dietary feathermeal inclusion level. Although,
464 harshly treated feed samples with a higher level of feathermeal were more prone to
465 oxidation and led to lower amino acid digestibility, the liver and plasma metabolites of the
466 fish appeared to be affected mainly by the feathermeal inclusion level. Furthermore, the
467 observed increase in certain AAs in the liver was presumably associated with energy
468 metabolism, suggesting a metabolic disturbance at the hepatic level that may explain the
469 higher FI and FCR in fish fed the highest FTH inclusion level. In comparison, a lower
470 level of feathermeal (FTH8) resulted in an increased SGR without any adverse effect on
471 FCR. To our knowledge, this is the first study correlating dietary protein oxidation effects,
472 amino acid digestibility, and liver and plasma metabolomics with growth performance of
473 fish as a means to explore the effects of replacing fishmeal with feathermeal in the diet.
474 The study demonstrated that the studied variables were useful as indexes for monitoring
475 fishmeal replacement with a new protein source in extruded fish feed. However, further
476 in-depth research is needed to determine the fate of oxidation products, metabolic

pathways, and pyruvate carrier inhibitors potentially affected by feathermeal included in extruded aquafeed.

ABBREVIATIONS USED

(FTH: feathermeal, AA: amino acids; FI: feed intake; SGR: specific growth rate; FCR: feed conversion ratio; AAR: amino acid racemization; PHP: Protein hydroperoxides; AU: arbitrary intensity units; FDAA: 2,4-Dinitro-5-fluorophenyl; ADC: apparent digestibility; TSP: sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate; PCA: Principal component analyses; CI: confidential interval; ANOVA: analysis of variance; NFK: N-formylkynurenine; ATP: Adenosine triphosphate; TCA: tricarboxylic acid; TMAO: Trimethylamine-n-oxide

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DECLARATION OF INTEREST

There is no conflict of interest.

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SUPPORTING INFORMATION

Following supporting information are available free of charge on the ACS Publications website at: DOI:

Metabolite differences in liver and plasma of fish fed the control diet (FTH0) and the diet with high inclusion of feathermeal (Table S1); Effect of different inclusion levels of feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130°C) on Schiff Base, *N*-Formylkynurenine, furosine, and racemization of amino acids (lysine, threonine, phenylalanine, and valine) in the different extrudates (Fig. S1); Effect of different extrusion temperatures (100 and 130°C) on differentiation of the studied observations based on protein and feed functional characteristics, and growth performance of the fish fed extruded feed containing different levels of feathermeal (Fig. S2); Effect of different extrusion temperatures (100 and 130°C) on differentiation of the studied observations based on growth response variables and metabolites from liver and plasma of the fish fed extruded feed containing different levels of feathermeal (Fig. S3)

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FIGURE CAPTIONS

Fig. 1: Effect of different inclusion levels of feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130 °C) on growth performance: feed intake (A), specific growth rate (B), feed conversion ratio (C) of rainbow trout after 8 weeks feeding trial with the different feeds, FTH0: 0% feathermeal, FTH8: 8% feathermeal, FTH24: 24% feathermeal. Results are expressed as mean ± SD and statistically significant differences between individual treatments assessed by Duncan test at $P < 0.05$ are indicated with different letters in superscript.

Fig. 2: Scores (A) and loading (B) plots based on extrudates and feed protein characteristics and fish growth performance for different levels of feathermeal, FTH0: 0% feathermeal, FTH8: 8% feathermeal; FTH24: 24% feathermeal. FCR: feed conversion ratio, SGR: specific growth rate, FI: feed intake. ◯: Amino acids digestibility; ☆: Oxidation (lanthionine (1), total carbonyls (2), Schiff base (3), N-formyl kynurenine (NFK) (4), protein hydroperoxides (PHP) (5), and furosine (6); ◇: Growth performance (FCR, FI, and SGR).

Fig. 3: Effect of different inclusion levels of feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130 °C) on protein hydroperoxides (A), carbonylation (B), lanthionine (C), and racemization of methionine (D) in the different extrudates, FTH0: 0% feathermeal, FTH8: 8% feathermeal, FTH24: 24% feathermeal. Results are expressed as mean ± SD, except for lanthionine in FTH0 and racemization of methionine in FTH8 extruded at 100 °C, which was measured once. Statistically significant differences between individual treatments assessed by Duncan test at $P < 0.05$ are indicated with different letters in superscript, except for those stated before.

Fig. 4: Scores (A) and loadings (B) plot based on growth response variables and metabolites from liver and plasma of the fish fed extruded feed containing different levels of feathermeal. FTH0: control fish fed feed with 0% feathermeal, FTH8: fish fed feed with 8% feathermeal; FTH24: fish fed feed with 24% feathermeal. ■: Plasma metabolites; ●: Liver metabolites; ◇: Growth Performance.

Table 1: Recipes and Nutrient Composition of Meal Mix, Extrudates, and Feed Including Three Inclusion Levels of Feathermeal (0, 8, and 24%)

Nutrient composition	FTH0 (g/100 g DM)	FTH8 (g/100 g DM)	FTH24 (g/100 g DM)
Recipe			
Fishmeal	52.57	42.84	20.67
Feathermeal		8.00	24.00
Wheat flour	19.70	21.00	24.07
L-Lysine HCI			1.08
DL-Methionine		0.01	0.37
L-Histidine	0.16	0.31	0.66
L-Tryptophan		0.01	
Mono-calcium phosphate			0.7
Yttrium	0.05	0.05	0.05
Fish oil*	6.40	6.40	6.50
Rapeseed oil*	19.10	19.30	19.50
Fishmeal			
Protein	71		
Lipid	10.30		
FTH			
Protein	86.40		
Lipid	6.90		
Meal mixes[a]			
Protein	51.20	53.60	52.60
Lipid	9.10	8.60	7.50
Extrudates[b]			
Protein	53.3±1.00	53.0±0.30	54.7±1.40
Lipid	9.50±0.20	8.90±0.10	7.60±0.10
Feed[c]			
Protein	39.9±0.50	39.7±1.50	40.2±0.70
Lipid	34.7±0.20	33.7±0.10	32.5±1.40
Total ash	8.95±0.07	7.55±0.07	5.40±0.00

DM: dry matter. FTH: feathermeal. *: oils used for coating the final feed. [a]sampled after meal mixer and before pre-conditioning. [b]sampled at the end of the extruder. [c]sampled after oil coating. The values for extrudates and feed are given as the mean±SD.

Table 2: m/z Values and Fragments Used for LC-MS/MS Analysis

Compound	**[M+H] *m/z***	**Fragments *m/z***
Deuterated lysine	151	88.1 and 134.1
Lanthionine	209	120.0
Lysinoalanine	234	130.1 and 84.1

Table 3: Effects of Extrusion Temperatures (100 and 130 °C) and Feathermeal Levels (0, 8, and 24%), and Their Interaction on Protein Oxidation and Growth Parameters of Fish Fed with the Experimental Feeds

	Temperature	FTH level	Temperature x FTH level
FCR	NS	*	NS
SGR	NS	*	*
FI	NS	*	*
PHP	*	*	*
Carbonyls	*	*	*
NFK	*	*	*
Schiff base	*	*	*
Lanthionine	NS	*	NS
Furosine	NS	NS	NS

FTH: Feathermeal, FCR: feed conversion ratio; SGR: specific growth rate; FI: feed intake; PHP: protein hydroperoxides; NFK: N-formylkynurenine. *: Significant at *P*-value <0.05, NS: not significant

Table 4: Significantly Different Absolute Concentrations of Metabolites (µmol/L) in Plasma of Rainbow Trout Fed the Experimental Diets with Different Inclusion Levels of Feathermeal (0, 8 and 24%) at Different Extrusion Temperatures (100 and 130 °C)

Metabolites	FTH0		FTH8		FTH24	
	100 °C	130 °C	100 °C	130 °C	100 °C	130 °C
Aromatic amino acid						
Phenylalanine	131 ± 14.7 ^{a}	119 ± 1.35 ^{a}	150 ± 13.7 ^{abc}	126 ± 6.06 ^{a}	152 ± 10.2 ^{b}	173 ± 14.5 ^{b}
Tyrosine	50.6 ± 2.87 ^{a}	51.2 ± 6.50 ^{a}	58.2 ± 19.1 ^{abc}	46.5 ± 10.4 ^{a}	68.8 ± 6.91 ^{b}	77.2 ± 4.79 ^{b}
Branched-chain amino acid						
Valine	472 ± 60.0 ^{a}	479 ± 5.84 ^{a}	575 ± 146 ^{abc}	528 ± 47.7 ^{ab}	712 ± 120 ^{c}	664 ± 19.0 ^{bc}
Other amino acid						
Aspartate	19.0 ± 3.48 ^{b}	16.2 ± 0.18 ^{ab}	13.5 ± 2.95 ^{ab}	20.0 ± 6.46 ^{b}	13.4 ± 3.23 ^{ab}	11.7 ± 2.21 ^{a}
Methionine	148.5 ± 21.7 ^{a}	145.2 ± 23.0 ^{a}	164.4 ± 42.1 ^{a}	142.5 ± 33.6 ^{a}	295.7 ± 55.6 ^{b}	354.3 ± 22.5 ^{b}
Glutamate	67.0 ± 31.3 ^{ab}	59.3 ± 25.5 ^{a}	126 ± 27.2 ^{b}	72.3 ± 22.9 ^{ab}	84.6 ± 52.8 ^{ab}	74.2 ± 10.2 ^{ab}
Organic acid and derivates						
Betaine	42.8 ± 1.96 ^{a}	60.3 ± 3.77 ^{ab}	58.9 ± 5.33 ^{ab}	61.1 ± 9.83 ^{ab}	79.1 ± 27.02 ^{b}	71.3 ± 9.62 ^{b}
Organic heterocyclic compounds						
Creatinine	128 ± 24.8 ^{cd}	180 ± 29.2 ^{d}	107 ± 36.7 ^{bc}	114 ± 53.1 ^{bc}	47.1 ± 1.37 ^{a}	58.7 ± 12.9 ^{ab}
N-Methylhydantoin	177 ± 23.0 ^{c}	173 ± 16.5 ^{c}	164 ± 25.5 ^{bc}	138 ± 6.54 ^{b}	83.7 ± 12.0 ^{a}	87.5 ± 10.6 ^{a}
Organic nitrogen compounds						
Dimethylamine	10.2 ± 1.51 ^{b}	11.8 ± 4.75 ^{b}	9.95 ± 2.31 ^{ab}	9.45 ± 5.07 ^{ab}	4.60 ± 0.69 ^{a}	5.05 ± 0.48 ^{a}
Trimethylamine	3.90 ± 0.60 ^{bc}	4.75 ± 0.09 ^{c}	3.20 ± 1.53 ^{abc}	3.45 ± 1.04 ^{abc}	2.00 ± 0.46 ^{a}	3.00 ± 0.54 ^{ab}
Trimethylamine N-oxide	24.9 ± 0.79 ^{c}	21.9 ± 1.66 ^{bc}	19.9 ± 5.68 ^{ab}	16.0 ± 4.61 ^{ab}	14.0 ± 2.80 ^{a}	17.2 ± 0.71 ^{ab}
Organic oxygen compounds						
Acetone	9.60 ± 1.19 ^{ab}	11.4 ± 2.5 ^{b}	8.75 ± 0.74 ^{a}	7.80 ± 0.84 ^{a}	7.85 ± 0.57 ^{a}	7.40 ± 0.48 ^{a}
Glucose	9719 ± 1138 ^{ab}	9198 ± 643 ^{a}	12367 ± 2103 ^{b}	10772 ± 1599 ^{ab}	13212 ± 2679 ^{b}	11432 ± 2170 ^{ab}
myo-Inositol	78.4 ± 29.2 ^{ab}	101 ± 34.9 ^{b}	55.6 ± 8.68 ^{a}	80.0 ± 13.6 ^{ab}	63.6 ± 21.7 ^{ab}	61.2 ± 14.3 ^{ab}
trans-4-Hydroxy-L-proline	88.0 ± 16.3 ^{ab}	93.3 ± 8.17 ^{ab}	106 ± 22.4 ^{b}	81.7 ± 15.1 ^{ab}	69.1 ± 10.9 ^{a}	86.7 ± 15.2 ^{ab}

Experimental diets: FTH0: fish fed control feed without feathermeal, FTH8: fish fed the 8% feathermeal diet; FTH24: fish fed the 24% feathermeal diet. Absolute concentration values are expressed as mean ± SD (n=21 per diet). ^{abcd}mean values across rows with different superscripts assessed by Duncan test are significantly different ($P < 0.05$)

Table 5: Significantly Different Absolute Concentrations of Metabolites (µmol/mg) in Liver of Rainbow Trout Fed the Experimental Diets with Different Inclusion Levels of Feathermeal (0, 8 and 24%) at Different Extrusion Temperatures (100 and 130 °C)

Metabolites	FTH0		FTH8		FTH24	
	100 °C	130 °C	100 °C	130 °C	100 °C	130 °C
Aromatic amino acid						
Phenylalanine	0.68 ± 0.04 ^{ab}	0.70 ± 0.09 ^{ab}	0.81 ± 0.18 ^{ab}	0.66 ± 0.05 ^{a}	0.86 ± 0.03 ^{b}	0.82 ± 0.09 ^{ab}
Branched-chain amino acid						
Isoleucine	0.78 ± 0.10 ^{a}	0.91 ± 0.14 ^{ab}	1.00 ± 0.11 ^{b}	0.80 ± 0.02 ^{a}	1.01 ± 0.06 ^{b}	0.86 ± 0.06 ^{b}
Leucine	1.88 ± 0.20 ^{ab}	2.17 ± 0.24 ^{ab}	1.98 ± 0.22 ^{ab}	1.79 ± 0.15 ^{a}	2.00 ± 0.26 ^{ab}	2.25 ± 0.28 ^{b}
Valine	1.28 ± 0.25 ^{a}	1.64 ± 0.18 ^{bc}	2.14 ± 0.28 ^{e}	1.46 ± 0.03 ^{ab}	2.07 ± 0.11 ^{dc}	1.80 ± 0.08 ^{cd}
Other amino acids						
Alanine	23.3 ± 2.44 ^{ab}	21.2 ± 2.69 ^{a}	22.3 ± 2.60 ^{ab}	24.7 ± 1.91 ^{ab}	27.2 ± 3.88 ^{b}	24.3 ± 3.03 ^{ab}
Creatine	1.57 ± 0.14 ^{ab}	1.68 ± 0.19 ^{b}	1.49 ± 0.09 ^{ab}	1.38 ± 0.18 ^{a}	1.42 ± 0.18 ^{ab}	1.32 ± 0.05 ^{a}
Glutamate	23.8 ± 3.72 ^{ab}	29.4 ± 4.78 ^{b}	23.6 ± 0.59 ^{ab}	23.5 ± 2.51 ^{a}	24.3 ± 3.11 ^{ab}	26.4 ± 0.57 ^{ab}
Lysine	2.11 ± 0.39 ^{ab}	2.65 ± 0.15 ^{ab}	1.81 ± 0.34 ^{a}	1.97 ± 0.23 ^{a}	1.66 ± 0.37 ^{a}	1.67 ± 0.31 ^{b}
Methionine	0.24 ± 0.03 ^{a}	0.49 ± 0.14 ^{b}	0.62 ± 0.18 ^{cd}	0.34 ± 0.08 ^{ab}	0.58 ± 0.11 ^{cd}	0.70 ± 0.02 ^{a}
Threonine	5.11 ± 0.81 ^{ab}	5.22 ± 0.42 ^{b}	4.57 ± 0.21 ^{ab}	5.05 ± 0.37 ^{ab}	4.65 ± 0.38 ^{ab}	4.25 ± 0.20 ^{a}
Nucleosides, nucleotides and analogues						
NADP+	0.19 ± 0.04 ^{b}	0.18 ± 0.02 ^{cd}	0.16 ± 0.02 ^{ab}	0.15 ± 0.03 ^{ab}	0.17 ± 0.01 ^{ab}	0.14 ± 0.01 ^{a}
UDP-glucose	1.55 ± 0.02 ^{d}	1.44 ± 0.12 ^{cd}	1.24 ± 0.17 ^{ab}	1.33 ± 0.07 ^{bc}	1.1 ± 0.05 ^{a}	1.31 ± 0.03 ^{bc}
UDP-glucoronate	1.74 ± 0.17 ^{b}	1.82 ± 0.04 ^{b}	1.55 ± 0.03 ^{a}	1.55 ± 0.05 ^{a}	1.58 ± 0.03 ^{a}	1.45 ± 0.07 ^{a}
Organic acid and derivates						
2-Aminobutyrate	0.21 ± 0.01 ^{ab}	0.29 ± 0.09 ^{b}	0.24 ± 0.03 ^{ab}	0.19 ± 0.04 ^{a}	0.18 ± 0.04 ^{a}	0.20 ± 0.01 ^{ab}
3-Hydroxyisobutyrate	0.15 ± 0.03 ^{ab}	0.17 ± 0.03 ^{ab}	0.17 ± 0.03 ^{ab}	0.14 ± 0.00 ^{a}	0.17 ± 0.01 ^{ab}	0.19 ± 0.04 ^{b}
5-Aminopentanoate	3.14 ± 1.00 ^{c}	2.56 ± 0.46 ^{abc}	1.87 ± 0.21 ^{ab}	2.68 ± 0.51 ^{bc}	2.20 ± 0.25 ^{abc}	1.63 ± 0.25 ^{a}
Formate	0.36 ± 0.09 ^{ab}	0.31 ± 0.05 ^{ab}	0.41 ± 0.07 ^{b}	0.29 ± 0.02 ^{a}	0.38 ± 0.03 ^{ab}	0.33 ± 0.06 ^{ab}
N,N-Dimethylglycine	0.03 ± 0.00 ^{abc}	0.04 ± 0.01 ^{c}	0.03 ± 0.01 ^{bc}	0.02 ± 0.00 ^{ab}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
Sarcosine	0.21 ± 0.09 ^{a}	0.06 ± 0.01 ^{ab}	0.04 ± 0.01 ^{a}	0.05 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}	0.06 ± 0.02 ^{a}
Taurine	141 ± 7.94 ^{b}	138 ± 3.13 ^{b}	136 ± 1.51 ^{b}	133 ± 5.33 ^{b}	134 ± 5.72 ^{b}	115± 9.34 ^{a}
β-Alanine	2.63 ± 0.45 ^{ab}	2.22 ± 0.37 ^{a}	2.95 ± 0.38 ^{bc}	2.49 ± 0.09 ^{ab}	3.55 ± 0.38 ^{c}	2.49 ± 0.33 ^{ab}
Organic heterocyclic compounds						
Creatinine	0.82 ± 0.24 ^{b}	0.85 ± 0.27 ^{b}	0.44 ± 0.05 ^{a}	0.60 ± 0.12 ^{ab}	0.52 ± 0.18 ^{ab}	0.39 ± 0.05 ^{a}
N-Methylhydantoin	0.59 ± 0.04 ^{d}	0.51 ± 0.10 ^{c}	0.42 ± 0.07^{b} ^{c}	0.45 ± 0.02 ^{bc}	0.39 ± 0.03 ^{b}	0.26 ± 0.04 ^{a}
Organic nitrogen compounds						
O-Phosphocholine	4.22 ± 0.72 ^{b}	4.09 ± 0.13 ^{ab}	3.04 ± 0.52 ^{ab}	4.28 ± 1.84 ^{b}	2.47 ± 0.70 ^{a}	2.67 ± 0.21 ^{ab}
Trimethylamine N-oxide	0.81 ± 0.22 ^{c}	0.62 ± 0.10 ^{abc}	0.43 ± 0.04 ^{a}	0.68 ± 0.15 ^{bc}	0.48 ± 0.04 ^{ab}	0.44 ± 0.04 ^{a}
Organic oxygen compounds						
Glucose	54.7 ± 5.07	69.7 ± 24.0 ^{b}	71.6 ± 13.0	41.0 ± 11.8 ^{a}	63.5 ± 9.83	51.2 ± 5.84

	ab	b	ab	ab

Experimental diets: FTH0: fish fed control feed without feathermeal, FTH8: fish fed the 8% feathermeal diet; FTH24: fish fed the 24% feathermeal diet. Absolute concentration values are expressed as mean ± SD (n=21 per diet). ^{abcd}mean values across rows with different superscripts assessed by Duncan test are significantly different (P<0.05)

Fig. 1:

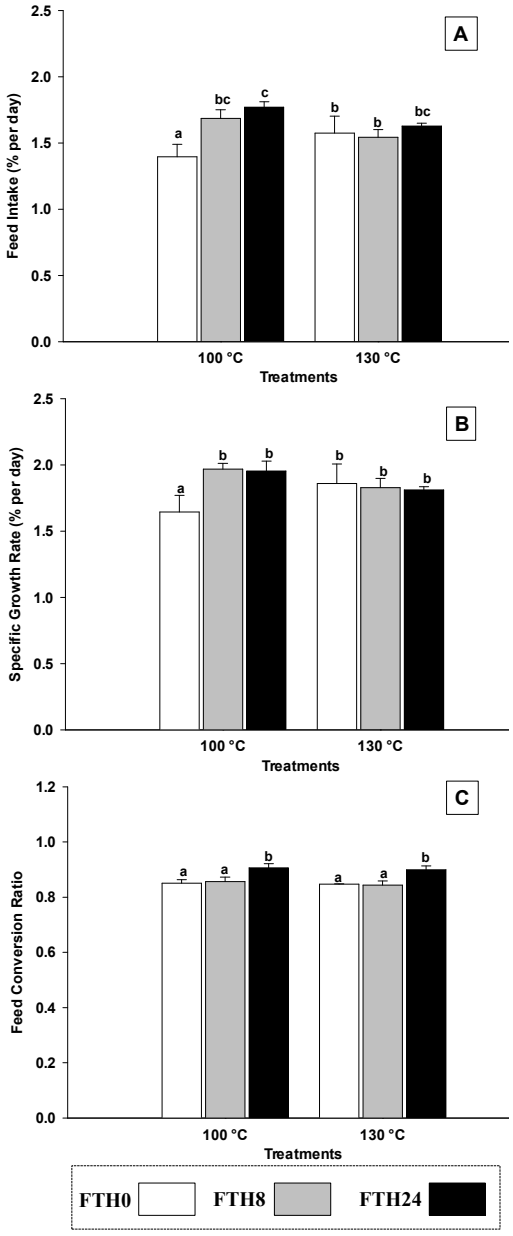

A
Feed Intake (% per day)
2.5
2.0
1.5
1.0
0.5
0.0
a
bc
c
b
b
bc
100 °C
130 °C
Treatments
B
Specific Growth Rate (% per day)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
b
b
b
100 °C
130 °C
Treatments
C
Feed Conversion Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
a
a
b
100 °C
130 °C
Treatments
FTH0
FTH8
FTH24

Fig. 2:

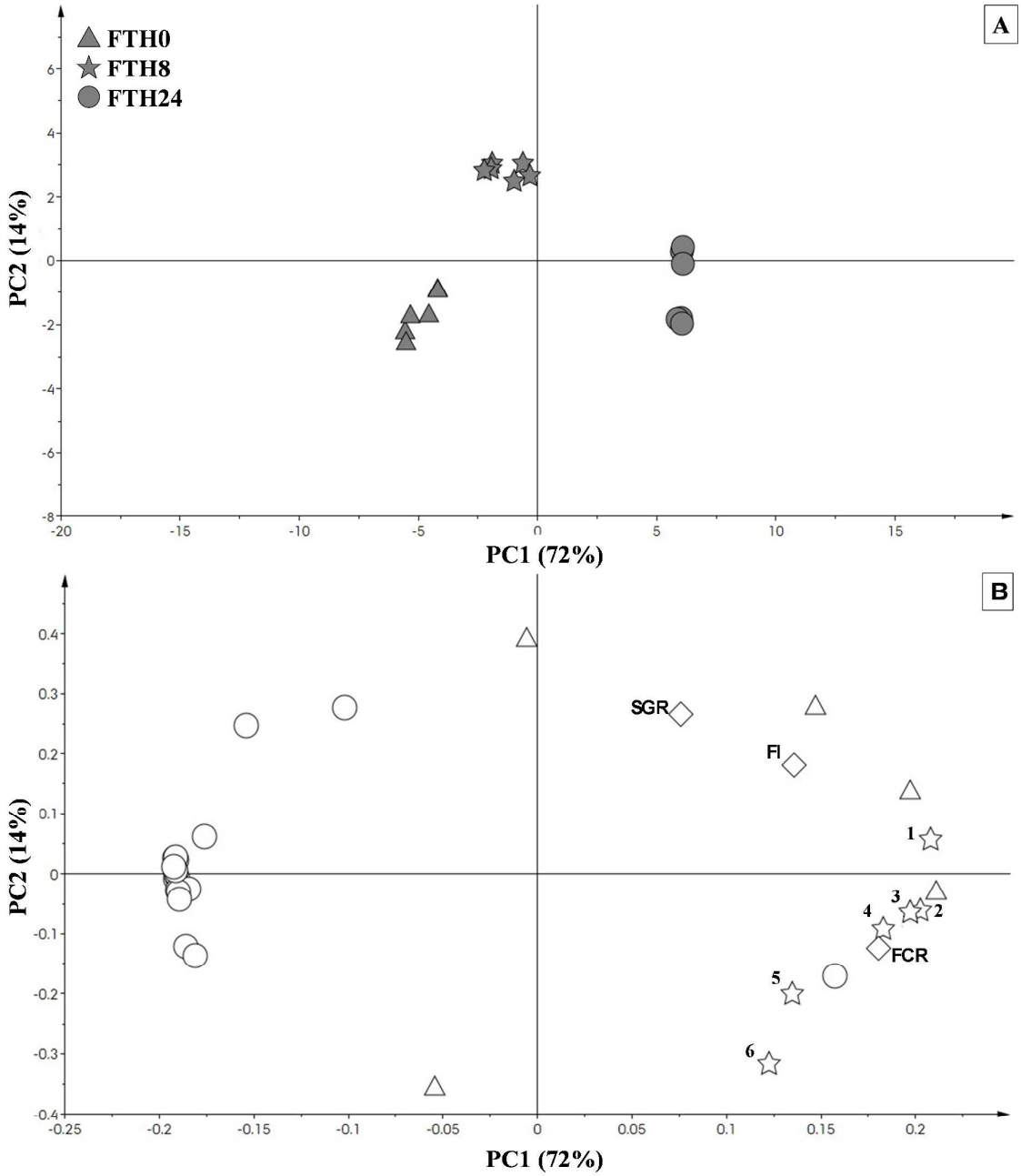


Fig. 3:

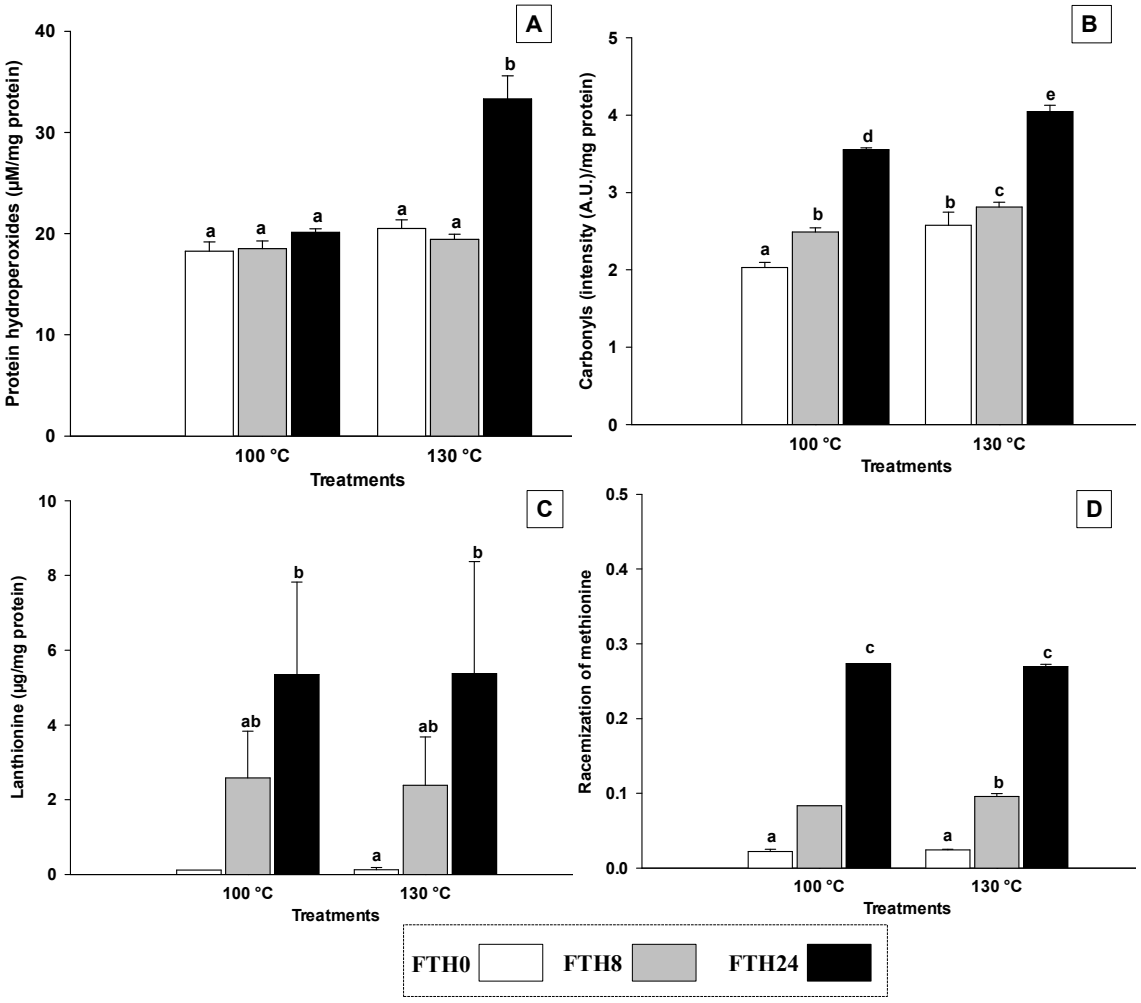
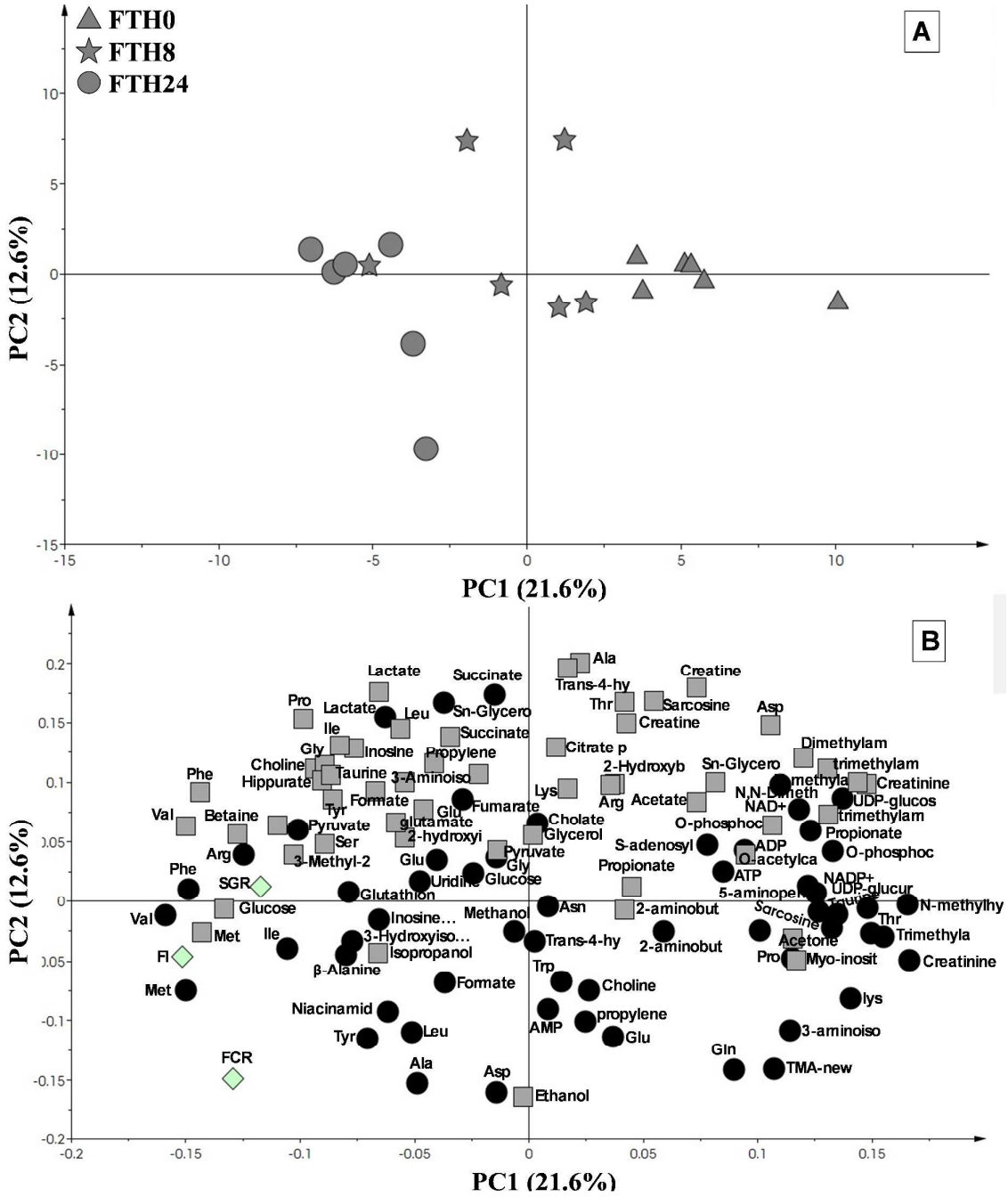
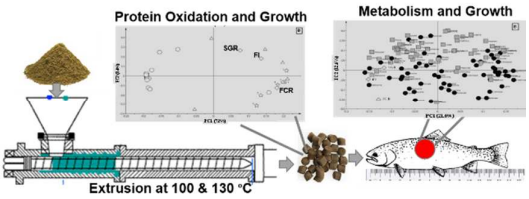

A
Protein hydroperoxides (µM/mg protein)
40
30
20
10
0
a
a
a
a
a
b
100 °C
130 °C
Treatments
B
Carbonyls (intensity (A.U.)/mg protein)
5
4
3
2
1
0
a
b
d
b
c
e
100 °C
130 °C
Treatments
C
Lanthionine (µg/mg protein)
10
8
6
4
2
0
ab
b
a
ab
b
100 °C
130 °C
Treatments
D
Racemization of methionine
0.5
0.4
0.3
0.2
0.1
0.0
a
c
a
b
c
100 °C
130 °C
Treatments
FTH0
FTH8
FTH24

Fig. 4:


FTH0
FTH8
FTH24
A
PC2 (12.6%)
PC1 (21.6%)
B
PC2 (12.6%)
PC1 (21.6%)
Lactate
Succinate
Ala
Creatine
Trans-4-hy
Thr
Sarcosine
Asp
Pro
Leu
Sn-Glycero
Ile
Inosine
Propylene
Citrate p
Dimethylam
Gly
Choline
Taurine
3-Aminoiso
2-Hydroxyb
trimethylam
Phe
Hippurate
Formate
Fumarate
Lys
Arg
Acetate
Creatinine
Tyr
Glu
Cholate
NAD+
UDP-glucos
Val
Betaine
Pyruvate
glutamate
Glycerol
O-phosphoc
Propionate
Ser
2-hydroxyi
S-adenosyl
ADP
Arg
3-Methyl-2
Pyruvate
O-acetylca
O-phosphoc
Phe
SGR
Uridine
Glucose
Propionate
ATP
NADP+
Glutathion
5-aminopen
UDP-glucur
Val
Glucose
Inosine…
Methanol
Asn
2-aminobut
N-methylhy
Met
Ile
3-Hydroxyiso…
Trans-4-hy
Thr
Fl
Isopropanol
2-aminobut
Acetone
Trimethyla
β-Alanine
Trp
Myo-inosit
Creatinine
Met
Formate
Choline
lys
Niacinamid
propylene
AMP
Tyr
Leu
Glu
3-aminoiso
FCR
Ala
Asp
Gln
TMA-new
Ethanol

TOC Graphic


Protein Oxidation and Growth
Metabolism and Growth
SGR
FI
FCR
Extrusion at 100 & 130 °C